

Identification of Interactions in the E1E2 Heterodimer of Hepatitis C Virus Important for Cell Entry*

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Several conserved domains critical for E1E2 assembly and hepatitis C virus entry have been identified in E1 and E2 envelope glycoproteins. However, the role of less conserved domains involved in cross-talk between either glycoprotein must be defined to fully understand how E1E2 undergoes conformational changes during cell entry. To characterize such domains and to identify their functional partners, we analyzed a set of intergenotypic E1E2 heterodimers derived from E1 and E2 of different genotypes. The infectivity of virions indicated that Con1 E1 did not form functional heterodimers when associated with E2 from H77. Biochemical analyses demonstrated that the reduced infectivity was not related to alteration of conformation and incorporation of Con1 E1/H77 E2 heterodimers but rather to cell entry defects. Thus, we generated chimeric E1E2 glycoproteins by exchanging different domains of each protein in order to restore functional heterodimers. We found that both the ectodomain and transmembrane domain of E1 influenced infectivity. Site-directed mutagenesis highlighted the role of amino acids 359, 373, and 375 in transmembrane domain in entry. In addition, we identified one domain involved in entry within the N-terminal part of E1, and we isolated a motif at position 219 that is critical for H77 function. Interestingly, using additional chimeric E1E2 complexes harboring substitutions in this motif, we found that the transmembrane domain of E1 acts as a partner of this motif. Therefore, we characterized domains of E1 and E2 that have co-evolved inside a given genotype to optimize their interactions and allow efficient entry.

Hepatitis C virus (HCV)⁴ is an important public health concern worldwide, as it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV is an enveloped

virus that belongs to the *Hepacivirus* genus of the Flaviviridae family (1). The two surface glycoproteins, E1 and E2, are processed by signal peptidases of the endoplasmic reticulum from a 3000-amino acid-long polyprotein encoded by the HCV genome (2).

Because of difficulties in propagating HCV in cell culture, many gaps remain in our understanding of the functions of E1 and E2. A major advance in the investigation of their functions was the development of HCV pseudoparticles (HCVpp) consisting of native HCV envelope glycoproteins E1 and E2 assembled onto retroviral core particles (3–5). Extensive characterization of HCVpp showed that they mimic the early steps of the HCV life cycle (6, 7). Furthermore, data obtained with HCVpp can now also be confirmed with the developed cell culture system that allows efficient amplification of HCV (HCVcc) (8–10).

The E1 (31 kDa) and E2 (70 kDa) proteins are glycosylated in their large N-terminal ectodomains and are anchored into the membrane by their C-terminal transmembrane domains. E1 and E2 form a heterodimer stabilized by noncovalent interactions that is retained in the endoplasmic reticulum (11). This oligomer was thought for a long time to be the prebudding form of the functional complex (12), which is present at the surface of HCV particles (13) and is involved in viral entry. Recent investigation of the E1E2 complex incorporated into HCVcc challenges this notion by proving the existence of large high molecular weight complexes stabilized by disulfide bridges (14).

HCV E2 is responsible for virion attachment to target cells and can bind different receptors including several capture molecules, the CD81 tetraspanin, and the scavenger receptor BI (for review, see Refs. 6 and 7). Recently, a three-dimensional structural model of E2 has been proposed as a class II fusion protein (15) based on the determination of its disulfide bonds which suggested that it can act alone to mediate binding and membrane fusion. However, both E1 and E2 appear to possess domains implicated in fusion (16–19). Moreover, several antibodies directed against E1 are able to neutralize cell entry, presumably at a stage distinct from receptor binding (20–22). Therefore, the role of E1 in HCV infection remains unclear.

The two transmembrane domains of the E1E2 heterodimer were shown to be important for different functions and interactions between the two glycoproteins. Studies of mutations occurring in conserved regions and analyses using cross-neutralizing antibodies have shown that these domains are involved in ER retention, heterodimerization of E1E2 on the

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⁴ The abbreviations used are: HCV, hepatitis C virus; tmd, transmembrane domain; HCVpp, HCV pseudoparticles; HCVcc, cell culture produced HCV; FFU, focus forming unit.

E1 Domains Interplay during HCV Cell Entry

surface of the viral particles, and even fusion between viral and cellular membranes (23–28).

The aim of this study was to characterize interactions between E1 and E2 and the cross-talk between these domains for conformational changes during entry. We assume that such domains of E1 and E2 will have co-evolved inside a given genotype to optimize their interactions and allow efficient entry. In this report we identified non optimal intergenotypic heterodimers that we used to identify less conserved domains involved in E1E2 interactions. We focused on E1E2 intergenotypic heterodimers between H77 (gt1a) and Con1 (gt1b) strains, and we generated chimeras in E1 by substituting H77 for Con1 sequences and vice versa to restore optimal entry function. We discovered that both the ectodomain and transmembrane domain are involved in the cross-talk, taking part during the conformational changes required for entry. Interestingly we show that the N terminus of E1, more precisely the AIL motif, and the transmembrane of E1 H77 need to be homogenous, which is to say from the same strain, to achieve optimal entry. This interaction is crucial for the entry of H77/JFH1 HCVcc chimera and seems to be genotype-dependent, as these interactions are not crucial for Con1. Thus, the specific interactions between E1 and E2 vary between strains.

EXPERIMENTAL PROCEDURES

Cell Lines—Huh-7 (29), Huh7.5 (30), and 293T (ATCC CRL-1573) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Perbio), 50 IU/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen).

Production of HCVpp—All chimeric E1E2 heterodimers have been constructed by PCR and/or digestion between genotype 1a strain H77 (31) (GenBankTM accession number AF009606) and 1b strain Con1 (32) (GenBankTM accession number AJ238799). All mutants were verified by sequencing. For infection assays and Western blots, HCVpp were produced as previously described (3) from 293T cells cotransfected with a murine leukemia virus (MLV) Gag-Pol packaging construct, an MLV-based transfer vector encoding the green fluorescent protein, and each of the E1E2 expression constructs. For Western blotting and co-immunoprecipitation assays, the pseudoparticles were purified and concentrated from the cell culture medium by ultracentrifugation at $82,000 \times g$ for 1 h 45 min at 4 °C through 1.5 ml of a 20% sucrose cushion. Viral pellets were suspended in phosphate-buffered saline (PBS) to concentrate the viral particles 100-fold. As a control for infection assays and co-immunoprecipitation assays, pseudoparticles devoid of viral glycoproteins were produced in parallel.

Incorporation of E1E2 Glycoproteins onto Viral Particles—Viral pellets were subjected to Western blot analysis using a mouse anti-HCV E1 antibody (IGH204) (Innogenetics), a mouse anti-HCV E2 antibody (H52) (33), and a goat anti-MLV-CA antibody (anti-p30; Viromed). Viral pellet samples were mixed with 6 \times loading buffer (375 mM Tris-HCl, pH 6.8, 3% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.06% bromophenol blue), and the samples were analyzed by electrophoresis in 12% polyacrylamide gels in the presence of 0.1% SDS. After protein transfer onto nitrocellulose filters, the blots were

blocked in Tris-buffered saline (1 M, pH 7.4) with 5% milk powder and 0.1% Tween 20 (34). The blots were probed with appropriate primary and secondary antibodies (1:10,000-diluted horseradish peroxidase-conjugated anti-mouse or anti-goat; Dako) in Tris-buffered saline, 5% milk, 0.1% Tween 20. Bound enzyme-labeled antibody was visualized using an enhanced chemiluminescence kit (SuperSignal West Pico chemiluminescent substrate; Pierce). To perform immunoprecipitation assays, the pelleted virions were lysed in immunoprecipitation buffer (20 mM Hepes, pH 7.5, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100), and the medium containing HCVpp was precleared by overnight incubation with a 1:1 mixture of protein A- and protein G-Sepharose beads (Amersham Biosciences) at 4 °C. After a centrifugation at $13,000 \times g$ at 4 °C for 5 min, the supernatants were incubated with the conformation-dependent anti-E2 monoclonal antibody AR3A (35) for 2 h at 4 °C, and the immune complexes were precipitated using a 1:1 mixture of protein A- and protein G-Sepharose beads for 1 h at 4 °C. The complexes were washed three times with immunoprecipitation buffer (50 mM NaCl, 50 mM Tris, pH 7.5, 10 mM EDTA) and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot using anti-E1 (IGH204) and anti-E2 (H52) antibodies.

Infection Assay—Supernatants containing HCVpp were harvested 36 h after transfection and filtered through 0.45- μ m-pore-size membranes. Huh-7 target cells (4×10^4 cells/well in 24-well plates) were incubated with different dilutions of HCVpp harboring the chimeric glycoproteins for 4 h at 37 °C. Supernatants were removed, and cells were incubated in complete medium for 72 h at 37 °C. Cells were detached and analyzed by FACS Canto II (BD Biosciences) for GFP expression.

CD81 Pulldown Assays—E1E2-transfected cells were lysed in pulldown lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 20 mM Imidazole, 2 mM Tris(2-carboxyethyl)phosphine, and 1% Triton X-100). Cell lysates were incubated or not with a recombinant protein containing the large extracellular loop of human CD81 fused to a His₆ tag (soluble CD81-LEL-His₆) overnight at 4 °C. Then, with nickel-nitrilotriacetic acid magnetic agarose beads and the BioSprint 15 Work station of Qiagen, the lysates were washed with NPI-20-T buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8) and eluted with a NPI-250-T buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8). Eluted samples were incubated with non-denaturing buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot using anti-E1 (IGH204), anti-E2 (H52), and anti-CD81 (JS81, BD Biosciences) antibodies.

Cell-Cell Fusion Assay—293T “donor” cells (2.5×10^5 cells/well seeded in six-well tissue culture dishes 24 h before transfection) were cotransfected using calcium phosphate reagent with 10 ng of E1E2 chimeric heterodimers and 20 ng of an HIV-1 long terminal repeat (LTR) luciferase reporter plasmid (a kind gift of Françoise Bex, Institut de Recherches Microbiologiques Jean-Marie Wiame) (36). As a negative control, cells were cotransfected with 10 ng of empty pCMV plasmid and 20 ng of the HIV-1-LTR-luciferase reporter plasmid. Twelve hours later, transfected cells were detached with Versene (0.53 mM EDTA; Invitrogen) and reseeded at the same concentration

(10^5 cells/well) in 6-well plates. Huh-7-Tat indicator cells (4×10^5 cells/well), detached with EDTA and washed, were then added to the transfected cells. After 24 h of cocultivation, the cells were washed with PBS, incubated for 5 min in a pH 5 fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES), and then washed 3 times with medium. The luciferase activity was measured 24 h later using a luciferase assay kit according to the manufacturer's instructions (Promega).

HCVcc Infection; *in Vitro* Transcription, HCVcc Production, Titration, and Viral Spread Kinetics—To generate infectious HCV RNAs, pJFH-1=S1a-NS2-1a2a VPL, termed H77/JFH1, and mutant were linearized at the 3' end by XbaI digestion and were treated with Mung Bean nuclease. Purified linearized DNAs were used as templates for *in vitro* transcription with the RiboMAXTM (Promega Corp.). *In vitro* transcribed RNA was used to electroporate Huh7.5 cells using Gene Pulser II apparatus (Bio-Rad) in an L3 laboratory, according to European safety regulations, and cells were cultured under standard conditions. Supernatant infectivity titers were determined as focus-forming units (FFUs) per ml. Huh7.5 cells were infected with different dilutions of culture supernatants. 2 days post-infection, FFUs were visualized after NS5A immunostaining as described previously (37). FFU calculations were based on counts of NS5A-positive cells. For the kinetic assays, producer cells were infected with a multiplicity of infection of 0.04. For virus spread, Huh7.5 producer cells were split and analyzed by FACS Canto II by NS5A immunostaining.

Quantitative Detection of HCV Core Protein by ELISA—HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany) according to the manufacturer's instructions.

RESULTS

The Functionality of Intergenotypic E1E2 Heterodimers for Entry Depends on the Compatibility of E2 with the E1 Genotype—To characterize the role of less conserved domains of the E1E2 heterodimer involved in cross-talk between either glycoprotein during their conformational changes in entry, we analyzed intergenotypic E1E2 complexes derived from E1 and E2 of different genotypes. The infectivity of HCVpp generated using E1 and E2 expressed in *trans* from individual plasmids indicated that H77 E1 (gt1a) forms functional heterodimers when associated with E2 derived from all genotypes tested from 1b, 2a, 3, 4, and 5 (Fig. 1A). Conversely, Con1 E1 (gt1b) does not form functional heterodimers when associated with E2 from H77 (gt1a) or JFH1 (gt2a) strains. Our study was focused on combinations using H77 and Con1 strains because of the use of antibodies against E1 (IGH204) and E2 (H52), which recognized linear epitopes on both strains. For a more natural expression context in producer cells, we decided to compare the results of E1E2 intergenotypic heterodimer for H77 and Con1 expressed in *cis*. These conditions allowed the production of equal amounts of E1 and E2 in HCVpp producer cells. Moreover, the HCVpp titers were increased 10-fold, improving the sensitivity of the assay as has been previously described (3, 38, 39). Western blot analysis on producer cells lysates did not indicate any difference in expression level (Fig. 1B). Furthermore, Western blot analy-

sis on purified HCVpp indicated that the level of Con1 E1 incorporated onto HCVpp harboring intergenotypic (heterogeneous) complexes (chimera #2) was similar to the quantity of E1 incorporated onto infectious HCVpp harboring the wild type (homogenous) Con1 complex. Similarly, H77 E2 was incorporated onto HCVpp harboring intergenotypic (heterogeneous) complexes (chimera #2) in a similar quantity to the H77 E2 incorporated onto infectious HCVpp E1E2 wt (homogenous) H77 (Fig. 1B). As with the *in trans* system, the expression of Con1 E1/H77 E2 in *cis* (chimera #2) from a polyprotein precursor still led to the production of HCVpp with a decreased titer compared with the wt homogenous combination or the H77 E1/Con1 E2 (chimera #4) mirror combination (Fig. 1D). Correlated with the biochemical analysis, these results indicated that the reduced infectivity observed with the Con1 E1/H77 E2 heterodimer (chimera #2) was not related to alterations of expression and incorporation of E1E2 heterodimers but rather to a cell entry defect (Fig. 1, B and D). Furthermore, compared with the mean 2-log decrease in titer in *trans*, we obtained a 1-log decrease in *cis*. This difference may be linked to the fact that the folding of the heterodimer is not optimal when E1 and E2 are expressed in *trans* as the folding of E1 and E2 are dependent on each other. Therefore, we decided to make subsequent chimera constructions in *cis*.

To further characterize the chimera Con1 E1/H77 E2, we wondered whether its non-optimal infectivity was linked to suboptimal recognition of the CD81 receptor. Using pulldown assays with soluble CD81-LEL harboring a His₆ tag, Western blotting analyses indicated that an equal quantity of E1 and E2 was co-immunoprecipitated, indicating that CD81 binding was not impaired in the different constructions. As a control, the input of CD81 precipitated was similar in all cases and E1E2 was not detected without soluble CD81 (Fig. 1C).

Both the Ectodomain and Transmembrane Domain of E1 Are Important for Heterodimer Functionality—To determine which domains are involved in the reduction of infection of the intergenotypic Con1 E1/H77 E2 heterodimer (chimera #2), we constructed chimeric E1 by substituting either the ectodomain or transmembrane domain (tmd) of H77 and Con1 strains, respectively. Biochemical analysis indicated that the different chimeric heterodimers had no alterations of expression and incorporation of E1E2 heterodimers (Fig. 2A). To ensure that the glycoproteins incorporated onto the particles were well associated on a conformational heterodimer, we carried out immunoprecipitation of E1E2 heterodimers from purified HCVpp using the AR3A conformational anti-E2 antibody. Western blotting analyses of precipitated complexes indicated that the different constructions displayed well-folded and associated E1E2 heterodimers on particles in similar proportions compared with the wild type heterodimers (Fig. 2B). To investigate further, pulldown assays with soluble CD81-LEL indicated that an equal quantity of E1 and E2 were co-immunoprecipitated, indicating that CD81 binding was not impaired in the different constructions (Fig. 2C). Therefore, the entry properties of chimeric heterodimers are not linked to conformation or assembly problems on the particles.

We next investigated the infectivity of the HCVpp harboring the different E1E2 chimeras generated (Fig. 2D). The titers of

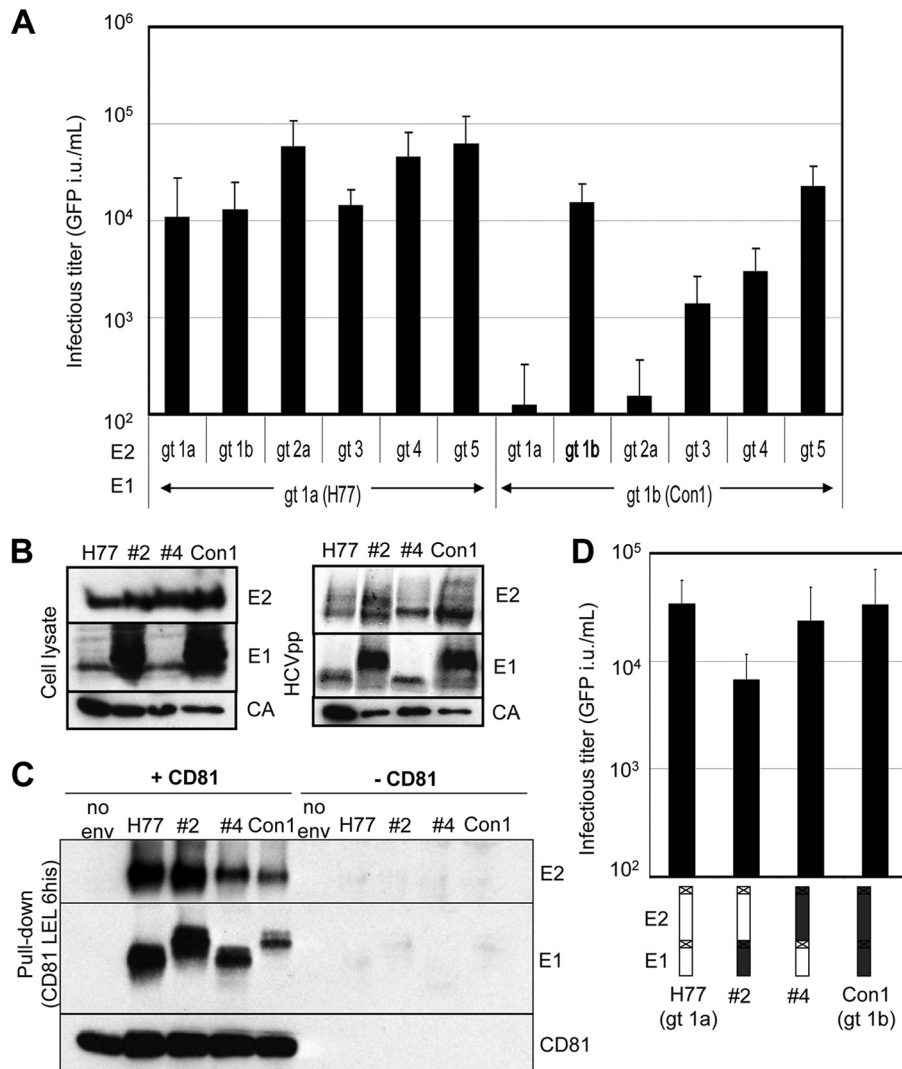


FIGURE 1. Cell entry property of HCVpp harboring E1E2 intergenotypic heterodimers. *A*, shown is infectivity of HCVpp using E1 from H77 (gt1a) or Con1 (gt1b) strains and E2 from 6 different strains (gt1a strain H77, gt1b strain Con1, gt2a strain JFH1, gt3 strain UKN3.1.9, gt4 strain UKN 4.11.1, and gt5 strain UKN 5.14.4) expressed in *trans*. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP-positive viable cells. The S.D. are the means of four experiments. *B*, expression and incorporation of E1E2 glycoproteins derived from different combinations of E1E2 heterodimers using E1 or E2 from H77 (gt1a) or Con1 (gt1b) expressed in *cis* onto HCVpp are shown. The expression in *cis* of the E1E2 glycoproteins was verified by Western blot of cell lysates of HCVpp producer cells using an anti-E1 antibody (IGH204), an anti-E2 antibody (H52), and an anti-capsid antibody (anti-p30, MLV-CA). The incorporation of the E1E2 envelope was analyzed by Western blot of viral particle pellets. Chimera #2 and #4 are the same as those described in *D*. *C*, shown is binding to CD81. The binding of E1E2 heterodimers to CD81 was analyzed by pull-down assays with (+CD81) or without (–CD81) soluble CD81-LEL-His₆ followed by Western blot using anti-E1 (IGH204), anti-E2 (H52), and anti-CD81 (JS81) antibodies. *D*, infectivity of the HCVpp derived from different combinations of E1E2 heterodimers using E1 or E2 from H77 (gt1a) or Con1 (gt1b) expressed in *cis*. The heterodimers are represented with schematic drawings. From bottom to top: E1 ectodomain, the TMD of E1 (cross), E2 ectodomain and TMD of E2 (cross). Black represents the domains that are from Con1 strain, and domains from H77 strain are in white. Infectious titers were determined as in *A*.

the different constructs indicated that the ectodomain was responsible for the loss of titer in the Con1 E1/H77 E2 combination (chimera #2). Indeed, compared with the titer of wild type H77, the introduction of the ectodomain led to a 2-log decrease (chimera #3). On the contrary, the substitution of the transmembrane of H77 E1 with the one of Con1 (chimera #1) did not modify the titer compared with the wild type H77. This may suggest that the tmd does not play a role in the function of intergenotypic heterodimer. However, when the titer of the Con1 E1/H77 E2 combination (chimera #2) was compared with the titer of the H77 harboring the sole ectodomain of Con1 E1 (chimera #3), there was a 1-log difference, which in this context did indicate a role for the tmd. Interestingly, when the mirror chimera was generated by introducing the sole ectodomain or

tmd of H77 E1 into the wt Con1 E1E2, the conclusion was similar. Indeed, even though the H77 E1/Con1 E2 combination (chimera #4) is optimal for entry, the introduction of either the transmembrane domain (chimera #6) or the ectodomain (chimera #5) alone leads to a 6- and 10-fold reduction in titer, respectively, compared with HCVpp harboring Con1 wt complex. Therefore, depending on the strain origin of E2, the full-length ectodomain and tmd have different roles in heterodimer functionality.

The Transmembrane Domain of E1 Is Essential for Entry—As the ectodomain and tmd are important for entry, we first decided to determine which amino acids in the transmembrane domains were responsible for the non-optimal functionality of chimera #3 and #5 observed in Fig. 2. Based on sequence align-

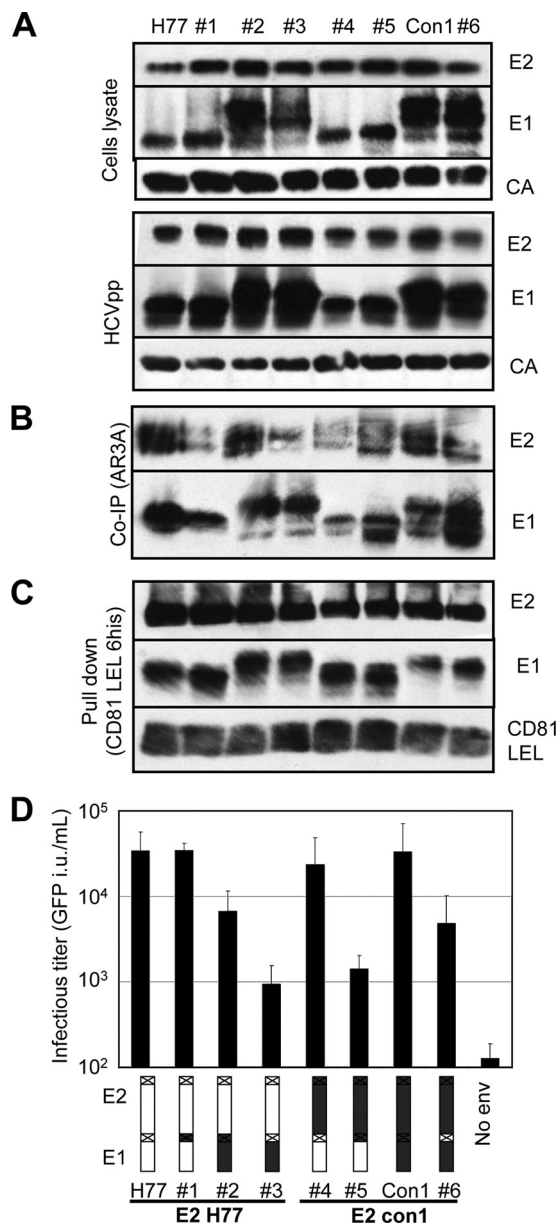


FIGURE 2. Properties of HCVpp harboring chimeric E1 in E1E2 heterodimers. A, expression and incorporation of E1E2 glycoproteins onto HCVpp are shown. The expression and the incorporation of the chimeric heterodimers were verified by Western blot as described in Fig. 1B. B, folding of E1E2 heterodimers is shown. The folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by co-immunoprecipitation of purified viral particles with the AR3A antibody, which recognizes a conformational epitope on E2, followed by Western blot of pellets using E1 (IGH204) and E2 (H52) antibodies. C, shown is binding to CD81. The binding of E1E2 heterodimers to CD81 was analyzed by pull-down assays as described before in Fig. 1C. D, infectivity of the HCVpp harboring chimeric E1 with H77 E2 or Con1 E2 is shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP-positive viable cells. The S.D. represents the means of four experiments. The heterodimers are represented as previously described in Fig. 1D.

ment analysis of the transmembrane domain of H77 and Con1, we found 4-amino acid differences at position 359, 362, 373, and 375 (Fig. 3A). To analyze their respective contribution, we first mutated each residue individually in the chimeric heterodimer Con1 harboring the ectodomain of H77 E1 (chimera #5) leading to the reintroduction of the H77 amino acid in the transmembrane domain. However, the single substitutions

L359I, Y362F, I373V, or M375L did not increase the titer of chimera #5 to the level of the fully infectious chimera #4 (Fig. 3B). We then generated different combinations of double and triple substitutions. Infection assays using these HCVpp indicated that three amino acid mutations are necessary to restore titer; L359I, I373V, and M375L (Fig. 3B). The expression and incorporation on HCVpp of the different heterodimers were verified by Western blot and were seen to be at equivalent levels (data not shown). The fact that incorporation is not affected is consistent with previous studies that demonstrated that these amino acids were not implicated in heterodimerization (26). As the transmembrane of Con1 seemed also to have a role in the functionality of chimeric heterodimers, we introduced the opposite substitution in the mirror chimeric heterodimer #3 (Fig. 3C). The H77 construct harboring the ectodomain of Con1 (chimera #3) was only weakly infectious, whereas the H77 E2 when associated with the Con1 E1 (chimera #2) was less affected (Fig. 3C), so we reintroduced Con1 amino acids into the H77 tmd of chimera #3. We obtained comparable results with heterodimers harboring the triple substitution I359L, V373I, and L375M, increasing the titer of chimera #3 (Fig. 3C). Therefore, regardless of the strain, the same three amino acids are important for infectivity of the HCVpp harboring chimeric heterodimers.

The N-terminal Motif Has a Genotype-dependent Role in HCV Entry Process—We next wondered which region of the H77 E1 ectodomain is needed for heterodimer functionality. Based on the sequence alignment of H77 and Con1 E1, we established different boundaries for the construction of chimeras with the 123 (chimera #7) or 36 (chimera #8) N-terminal residues from Con1 in the H77 E1 (Fig. 3A). As for other chimeras, biochemical analysis indicated that expression, incorporation, conformation, and CD81 binding capacity were similar for each construction (Fig. 4, A–C). Infection assays using HCVpp harboring these chimeric heterodimers indicated that residues included in the 36 N-terminal amino acids of H77 were necessary for optimal infection of H77 (Fig. 4D). The repertoire of the residues in this N-terminal domain (data not shown) indicated that two different motifs are particularly heterogeneous between the different genotypes; that is, the SNA/PNS (208–210) and the MIM/AIL (219–221) motifs in Con1/H77, respectively (Fig. 3A). To establish which of these motifs were crucial for H77 heterodimer function in entry, we restored these motifs in the non-optimal H77 chimeric heterodimer harboring the 36-N-terminal domain from Con1 (chimera #8). On one hand, biochemical analysis indicated that the difference of titer was not linked to a defect in expression, assembly, conformation, or binding to CD81 (Fig. 5, A–C). On the other hand, the results of infection assays indicated that the substitution of the SNA motif to PNS was not able to restore the wild type titer, whereas the substitution of the MIM motif to AIL was sufficient to restore an infectivity closely similar to wt H77 (Fig. 5D). To further verify this, we introduced a single mutation in the defective heterodimer (chimera #8) and showed that the M219A substitution was sufficient to restore optimal infectivity. On the contrary, Leu-221 did not play a role in H77 heterodimer functionality (Fig. 5D). Therefore, Ala-219 seemed critical for the functionality of the H77 heterodimer. To confirm this hypoth-

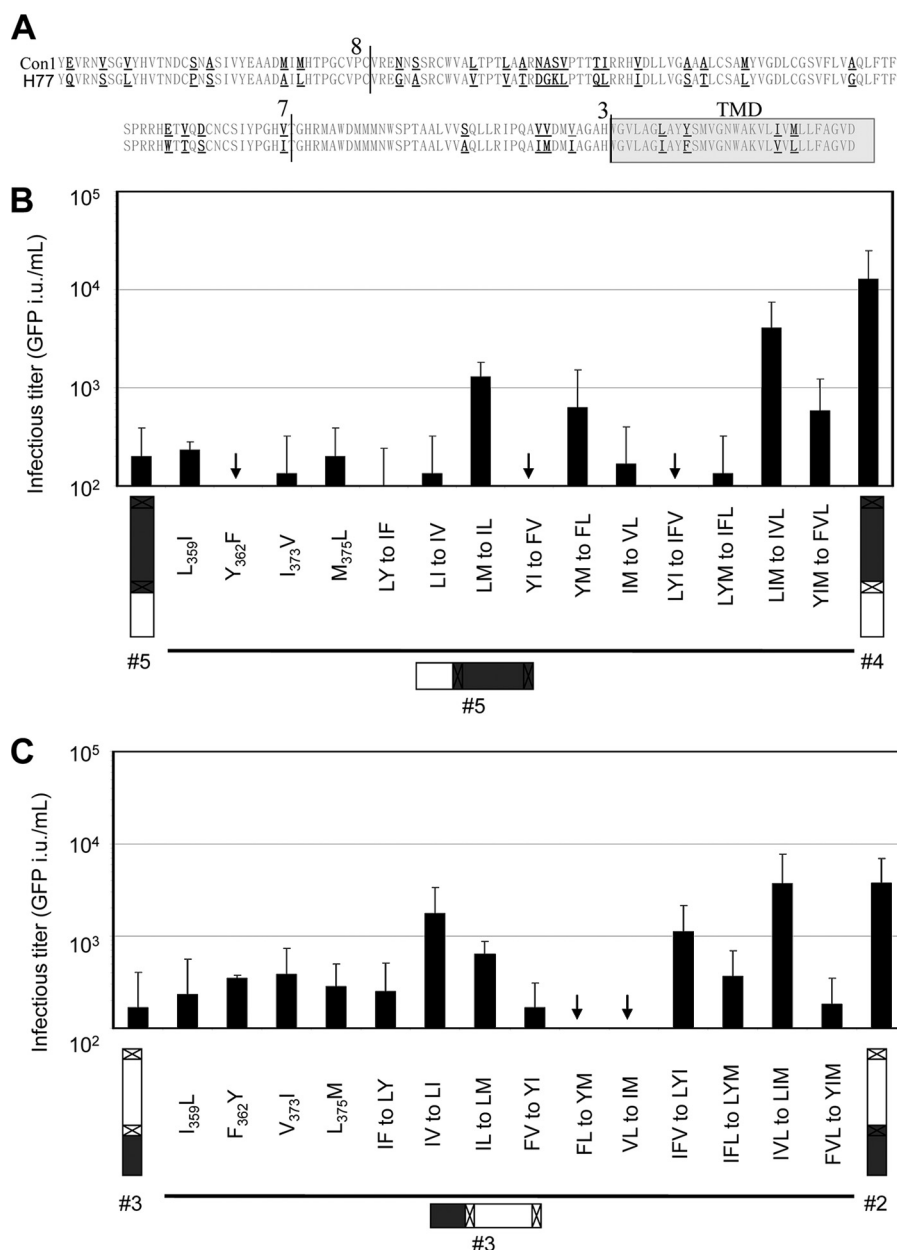


FIGURE 3. Properties of HCVpp harboring mutations in the E1 transmembrane domain. A, shown is a comparison of E1 sequences from H77 and Con1 strains. In the sequence alignment, the differences are represented in **bold**. The point of chimerization is represented and denoted by the number referring to the name of chimeras. The transmembrane domain (TMD) is boxed in gray. B, shown is infectivity of the HCVpp harboring mutations in the E1 tmd of chimera #5, which reintroduce H77 amino acids. C, infectivity of the HCVpp-harboring mutations in the E1 tmd of chimera #3, which reintroduce Con1 amino acids, is shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP positive viable cells. The S.D. are the means of four experiments. The heterodimers are represented as previously described in Fig. 1D.

esis, we introduced a mutation in this motif in H77 E1E2 or Con1 E1E2. As expected, the titer of HCVpp harboring H77 E1E2 with the MIM motif was decreased by 1 log compared with wt H77 (Fig. 5E, *black bar*). On the contrary, the introduction of the AIL motif in Con1 E1E2 induced no decrease of titer compared with wt Con1 (Fig. 5E, *black bar*). Altogether, these results indicate that the non-optimal titer of HCVpp induced by the introduction of the MIM motif or A219M substitution in the H77 E1 N terminus is due to a genotype-dependent entry defect.

Because our results indicated a normal capacity of the mutant proteins to mediate CD81 binding (Fig. 5C), the loss in

infectivity of the H77 E1E2 MIM mutant could be due to a defect in the membrane fusion process. To address this point, we performed cell-cell fusion (syncytium) assays (16) whereby 293T donor cells, expressing a luciferase marker gene under the control of the HIV-1 promoter, were cocultured with Huh-7-Tat "indicator" cells, expressing the HIV-1 transactivator of transcription (Tat) protein. Because the HIV-1 promoter requires Tat for efficient expression, only fused cells should express detectable levels of luciferase. Donor cells were transfected with expression plasmids encoding wild type or mutant E1E2 glycoproteins. The results were in agreement with the results of infection assays. Compared with wt H77, mutants

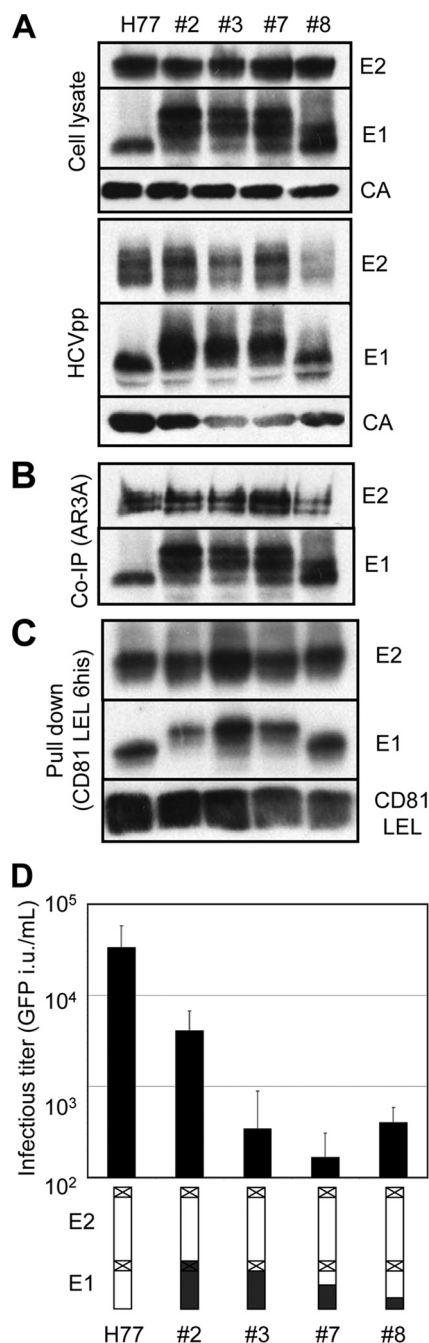


FIGURE 4. Properties of HCVpp harboring E1E2 chimeric E1 ectodomain in H77 heterodimer. *A*, expression and incorporation of E1E2 glycoproteins onto HCVpp is shown. The expression and incorporation of the chimeric heterodimers were verified by Western blot as described in Fig. 1*B*. *B*, folding of E1E2 heterodimers is shown. The folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by co-immunoprecipitation (Co-IP) as described before in Fig. 2*B*. *C*, binding to CD81 is shown. The binding of E1E2 heterodimers to CD81 was analyzed by pull-down assays as described before in Fig. 1*C*. *D*, infectivity of the HCVpp-harboring chimeric ectodomain of E1 with H77 E2 is shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP positive viable cells. The S.D. are the means of four experiments. The heterodimers are represented as previously described in Fig. 1*D* and 3*A*.

H77 E1E2 with the MIM motif had reduced cell-cell fusion activity (Fig. 5*E*, *gray bar*), concomitant with decreased infectious titers (Fig. 5*E*, *black bar*). On the contrary, no significant differences in cell-cell fusion were measured between wt Con1

and mutant Con1 E1E2 with AIL motif (Fig. 5*E*, *gray bar*). Thus, the results of cell-cell fusion assays indicated that the mutant H77 E1E2 with the MIM motif is impaired in its capacity to mediate membrane fusion.

To confirm these results in replicative HCVcc, which affords a more relevant model of assembly, we introduced this substitution into an H77/JFH1 HCVcc chimeric construct harboring E1E2 from strain H77. Transcribed RNA was electroporated into Huh7.5, and the titers of virus released into the cell culture supernatants were measured 2 days post-electroporation. We verified that the producer cells were electroporated at equivalent levels by NS5A immunostaining and FACS analyses to ensure that the difference of titer was not caused by a different level of electroporation and initial expression (Fig. 5*F*, *white bar*). The titer of HCVcc harboring H77 with the MIM motif was 10-fold lower than the titer of HCVcc carrying the wt H77 E1E2 (Fig. 5*F*, *black bar*). However, using core ELISA assays, we showed that the quantity of core released into the cell culture supernatant was similar, even slightly higher for HCVcc harboring the mutated MIM H77 E1E2 (Fig. 5*F*, *gray bar*). This result strongly suggests that the difference in titers observed between HCVcc harboring wt H77 E1E2 and mutated MIM H77 is due to an entry defect rather than an assembly problem. HCVcc harboring the mutated MIM H77 E1E2 has a reduced titer linked to an inhibition of viral growth and production of viral particles (Fig. 5*G*). Indeed, whereas H77 HCVcc propagate in cell culture after low multiplicities of infection (0.04), as shown by infectious titer in the supernatant (Fig. 5*G*, *left panel*), by the percentage of infected cells measured with NS5A-positive cells (Fig. 5*G*, *middle panel*), or by the quantity of core released into the supernatant (Fig. 5*G*, *right panel*), the HCVcc harboring the mutated MIM H77 E1E2 displayed much slower propagation rates. This is in agreement with the infectivity defect of HCVcc after electroporation (Fig. 5*F*) and of HCVpp (Fig. 5*E*). Altogether, these data indicate that the role in entry of the N-terminal motif AIL/MIM is essential for the H77 strain.

The N-terminal Domain and the Transmembrane Domain of H77 Interact for Entry—Having determined that three amino acids are important in the E1 transmembrane and that the E1 N-terminal AIL/MIM motif is critical at least in H77 E1, we decided to test the impact of the association of the full-length heterogeneous transmembrane domain with the AIL/MIM motif in the constructions described in Fig. 2. We, therefore, substituted the AIL for MIM or MIM for AIL motif in the chimeric heterodimers that contain the ectodomain and/or transmembrane domain of E1 from the two different strains (Fig. 6). Biochemical analysis indicated again that differences of titer were not linked to expression, incorporation, or conformation of E1E2 on HCVpp (Fig. 6, *A* and *B*). It should be noted, however, that the quantity of E2 and E1 co-immunoprecipitated using the AR3A anti E2 antibody is slightly lower for chimera #4 and #4MIM, chimera #5 and #5MIM, Con1 and Con1AIL, and chimera #6 and #6AIL, than for the other chimeras (Fig. 6*B*). This difference might be due mainly to a different affinity of AR3A antibody for E2 H77 and E2 Con1, leading to a weaker co-immunoprecipitation of the chimera harboring a Con1 E2 than H77 E2 rather than major conformation differences induced by mutations. A difference was also detected in the

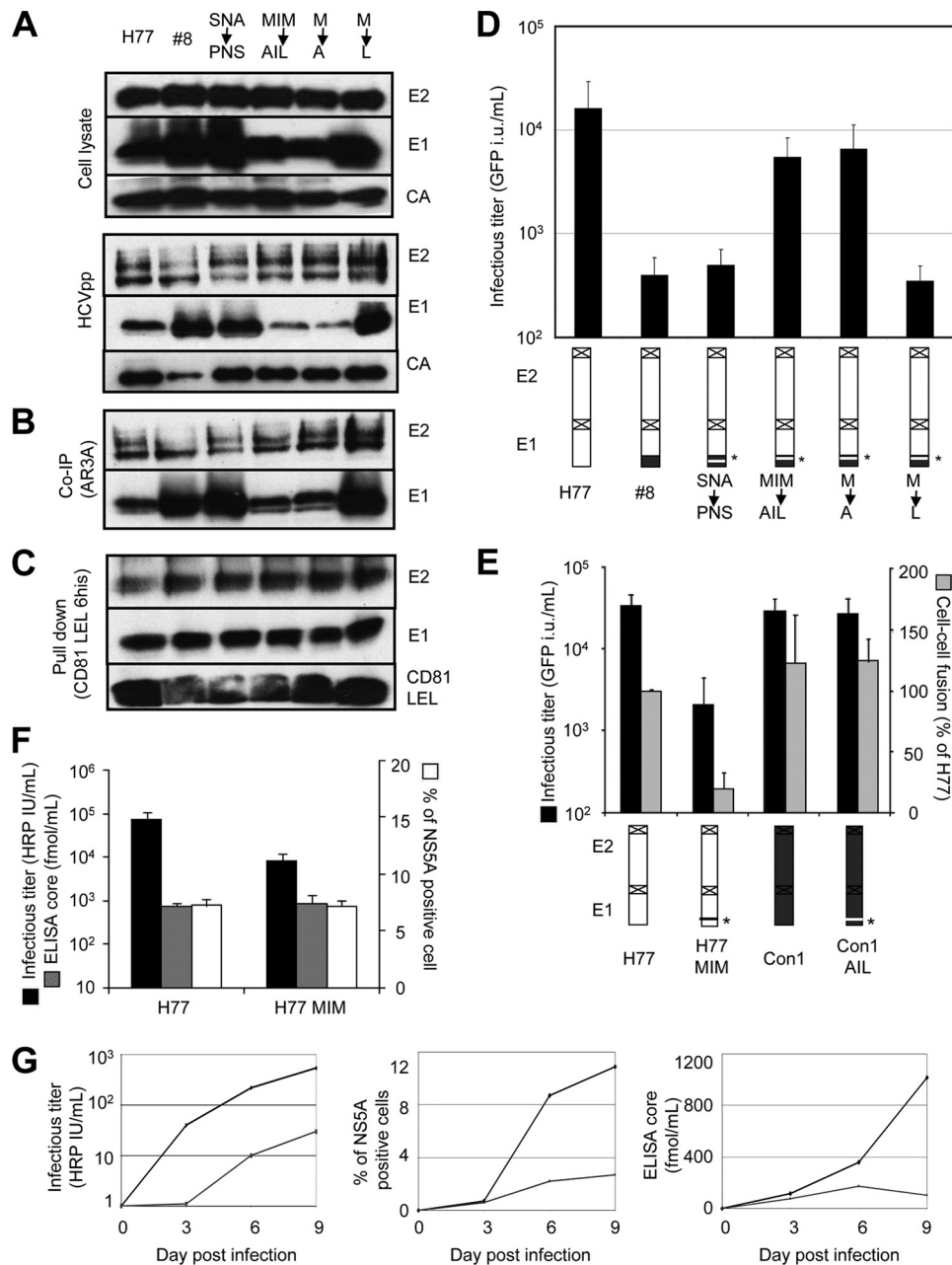


FIGURE 5. Properties of HCVpp and HCVcc-harboring mutations in the N-terminal chimeric E1. A, shown is expression and incorporation of E1E2 glycoproteins onto HCVpp. The expression and the incorporation of the chimeric heterodimers were verified by Western blot as described in Fig. 1B. B, folding of E1E2 heterodimers is shown. The folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by co-immunoprecipitation (Co-IP) as described in Fig. 2B. C, shown is binding to CD81. The binding of E1E2 heterodimers on CD81 was analyzed by pull-down assays as described in Fig. 1C. D, infectivity of the HCVpp harboring mutations in the N-terminal chimeric ectodomain of E1 with H77 E2 is shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP-positive viable cells. The S.D. are the means of four experiments. The heterodimers are represented as previously described in Fig. 1D. The white lines with an asterisk symbolize the mutation of the different motifs. E, infectivity of HCVpp and cell-cell fusion assays with H77 and Con1 E1E2 heterodimers with mutations in the AIL/MIM motif are shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP-positive viable cells. Cell-cell fusion assays were represented as the percentage of fusion compared with the E1E2 H77 heterodimer. The S.D. are the means of four experiments. The heterodimers are represented as previously described in Fig. 1D. The white or black lines with an asterisk symbolize the mutations of the motif. F, impact of the AIL motif in H77/JFH1 HCVcc construct for entry is shown. Two days post-electroporation, infectivity (in black), quantity of core protein in the supernatant (in gray) and level of electroporation (in white) were studied. Huh7.5 cells were infected with different dilutions of culture supernatants. Supernatant infectivity titers were determined as FFUs/ml based on counts of NS5A-positive cells in focal immunoassay. Quantity of Core (in fmol/ml) in supernatant was measured by ELISA Core for each construct. Levels of electroporation were detected by FACS by NS5A immunostaining of electroporated cells and are represented as the percentage of positive cells. The S.D. are the means of four experiments. G, kinetic assays for H77/JFH1 HCVcc constructs are shown. Wt H77 E1E2 is represented in black and mutated H77MIM in gray. Producer cells were infected with a multiplicity of infection of 0.04 on day 0 and analyzed every 3 days over 9 days for infectious titers (left graph), virus spread (middle graph), and quantity of core in the supernatant (right graph). Supernatant infectivity titers were determined as in F. For virus spread, Huh7.5 producer cells were split and analyzed by FACS by NS5A immunostaining as in F. Quantity of core (in fmol/ml) in supernatant was measured by ELISA Core for each construct.

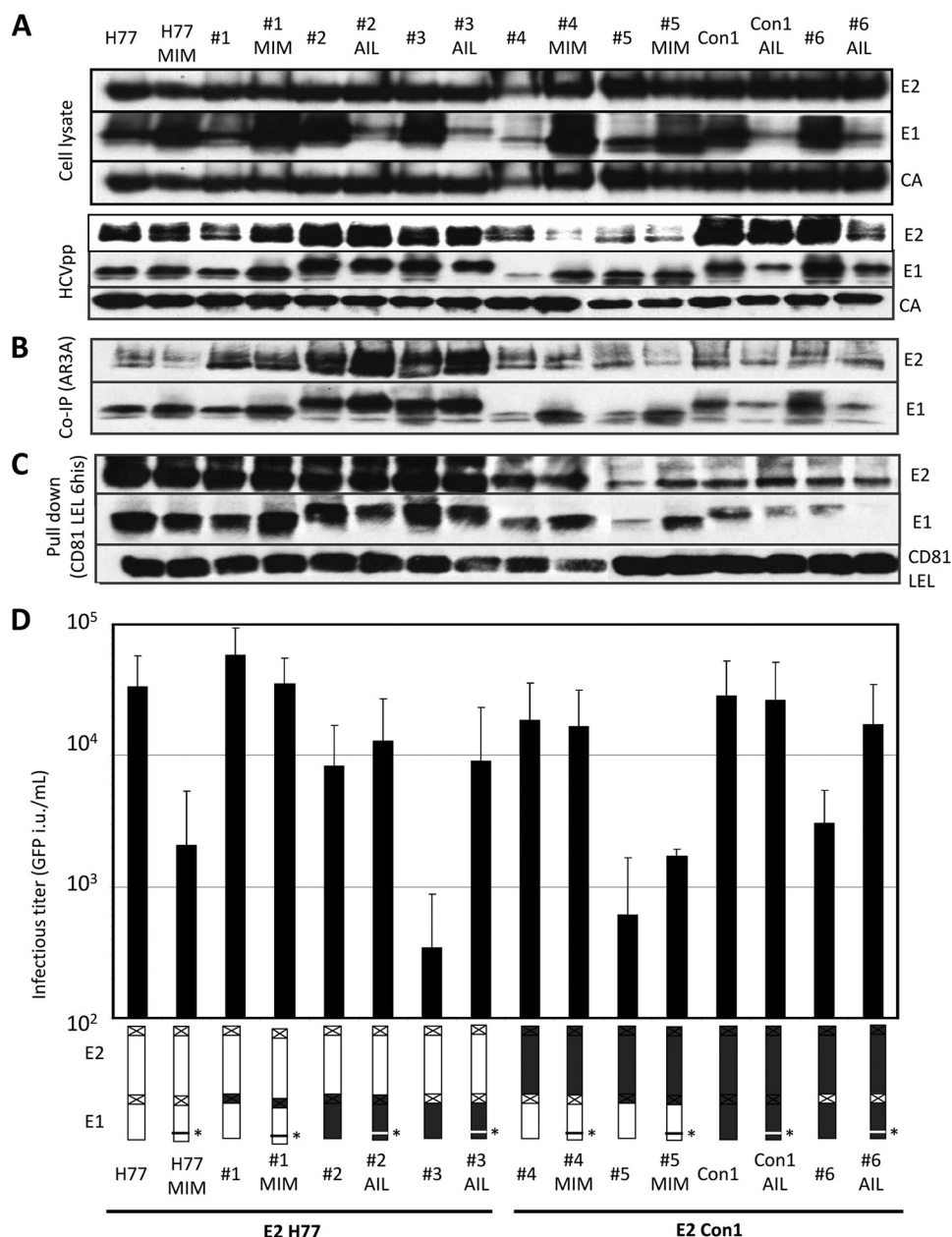


FIGURE 6. Properties of HCVpp harboring AIL or MIM mutations in chimeric E1E2 heterodimers for ectodomain or tmd of E1. *A*, expression and incorporation of E1E2 glycoproteins onto HCVpp are shown. The expression and the incorporation of the chimeric heterodimers were verified by Western blot as described in Fig. 1*B*. *B*, folding of E1E2 heterodimers is shown. The folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by co-immunoprecipitation (Co-IP) as described in Fig. 2*B*. *C*, binding to CD81 is shown. The binding of E1E2 heterodimers on CD81 was analyzed by pull-down assays as described in Fig. 1*C*. *D*, infectivity of the HCVpp harboring chimeric heterodimers is shown. The infectious titers were deduced from the transduction efficiencies, determined as the percentage of GFP-positive cells. The S.D. are derived from the means of four experiments. The heterodimers are represented as previously described in Fig. 1*D*. The white or black lines with a star symbolize the mutation of the different motifs.

quantity of H77 E2 and Con1 E2 recognized by CD81, which probably reflects the difference in CD81 binding of the two genotypes as previously described (40). However, when the quantity of E1 or E2 detected with the different combinations was compared with the wt E1E2 combination harboring the same E2 strain, no major differences were observed between the different combinations (Fig. 6*C*).

Interestingly, when the constructs contained the transmembrane domain of Con1, the titers were the same regardless of whether the MIM or AIL motif was present (compare chimera #1 and #1MIM, chimera #2 and #2AIL, chimera #5 and #5

MIM, Con1 and Con1AIL). However, for the construction containing the E1 transmembrane domain of H77, the titer was improved when associated with AIL motif, which restored the homogenous combination (compare chimera #3 and #3AIL, chimera #6 and #6AIL, H77MIM and H77). The only exception was the heterodimer H77 E1/Con1 E2 (chimera #4), which was still fully functional when the MIM motif was present (Fig. 6*D*). These results suggest a compensative interaction between H77 E1 ectodomain and Con1 E2 that could counteract the defective interaction of the E1 transmembrane domain of H77 with the Con1 MIM motif.

Altogether, these results strongly suggest that the interaction of the transmembrane of E1 H77 and the AIL motif are necessary for entry but not for heterodimerization and CD81 binding. It must be noted that this interaction can be counteracted by other potential interactions between E1 and E2.

DISCUSSION

To identify domains implicated in the HCV entry process that have co-evolved in a given genotype, we generated intergenotypic E1E2 heterodimers. We identified intergenotypic incompatibility between E1 and E2 that led to partial loss of entry function in certain combinations, namely, when Con1 E1 is associated with E2 from H77 (as in chimera #2) or JFH1. Entry defect was not due to the lack of heterodimerization or the lack of CD81 interaction. Focusing on genotype H77 and Con1, we determined that the contributions of the transmembrane domain and ectodomain of E1 could not be separated, and we identified 2 non-conserved regions that played a role in interaction within E1. More precisely, we identified the N-terminal motif AIL/MIM (219–221) and 3 amino acids, previously not considered important (359, 373, and 375) in the transmembrane domain, as necessary for optimal infectivity. Interestingly, the cross-talk between the N-terminal E1 motif (AIL/MIM) and the transmembrane domain of E1 seems to be important only for H77 E1E2 functionality. Indeed, HCVpp harboring chimeras with Con1 E1 tmd resulted in the same level of infection for both AIL/MIM motifs. However, most of the HCVpp harboring chimeras with H77 E1 tmd gave a lower titer when associated to the MIM (Con1) motif compared with the AIL (H77) motif. Therefore, we showed that it is essential for certain domains to be homogeneous (*i.e.* from the same HCV strain) as they have co-evolved within a given HCV genotype to achieve interrelations for optimizing cell entry functions. Interestingly, our work is complementary to a recent study (41) that used a similar strategy to identify that E1 JFH1 (gt2a) does not form functional heterodimers when associated with E2 from H77 (gt1a). This study demonstrated intradomain interactions within E2 but did not focus on E1.

Besides N-terminal and tmd Cross-talk in E1, E2 Is Involved in Another Interaction—Despite confirming a function of cross-talk between E1 and E2 domains, we were also able to make a detailed characterization of interactions inside E1. This result is most likely linked to the fact that there was no reciprocity in the identified non-optimal combination. Indeed, whereas Con1 E1 did not tolerate H77 E2 (chimera #2) or JFH1 E2 for optimal infectivity, E1 from H77 tolerates all E2, including Con1 E2 (chimera #4). This alone indicates that some aspects of cross-talk between E1 and E2 are not identical, depending on the genotypes and the E1 considered. However, it was surprising to observe that when only the ectodomain was exchanged, the reciprocity was verified. Indeed, the Con1 E1 ectodomain did not tolerate H77 sequences (chimera #3) and neither did the H77 E1 ectodomain tolerate Con1 sequences (chimera #5). However, the mechanism of non-functionality of these chimeras might be different.

Based on our results, we suggested that the tmd of Con1 can tolerate both AIL/MIM motif (in Fig. 6D, compare chimera #2 and #2AIL, Con1 and Con1AIL, chimera #1MIM and #1, chi-

mera #5MIM and #5). However, the association of the H77 E1 ectodomain with Con1 sequence (chimera #5) was not functional regardless of the N-terminal motif. Therefore, in this case, there is probably another interaction involved in the defect of the infectivity that may involve E2 and/or another domain in E1.

Similarly, we suggested that the compatibility between the H77 E1 tmd and the N-terminal motif AIL is important for H77. In this respect, when H77 E1 tmd is associated with the AIL motif, the infectivity of HCVpp is optimal (chimeras #3AIL, #6AIL, and H77). The exception to this observation is the intergenotypic heterodimer H77 E1/Con1 E2 (chimera #4), which is functional with both motifs. There are, again, probably other interactions involved in the optimization of infectivity.

As our theory does not apply for two chimeras (#4 and #5), the analyses should take into account the origin of E2. Indeed, for these two chimeras, the E2 is derived from Con1 (that does not tolerate all E1). Because of these discrepancies with the model of interaction between the N-terminal motif and the tmd in E1 (which are validated by all the constructs harboring E2 H77), another level of interaction involving E2 should be included. Our results also indicated different characteristics between chimeras harboring H77 E2 or Con1 E2. Indeed, chimeras harboring Con1 E2 are less efficient for CD81 binding. This differential characteristic for receptor binding depending on E1E2 strains has already been studied. CD81 and scavenger receptor BI can interact with E1E2 heterodimer differently depending on HCV strains (40, 42–45). All these differences underlined that E1E2 functionality for entry can differ between genotypes and even strains. Hence, the understanding of the precise molecular mechanisms of E1E2 during entry might be difficult to generalize to all HCV strains.

Therefore, we propose that an interaction inside E1 is important for the optimal functionality of HCV envelope glycoproteins (see interaction 1 in Fig. 7), but another interaction between E1 and E2 also appears to be important (see interaction 2 in Fig. 7). However, in our analysis, we were unable to more precisely uncover this second interaction. One possibility will be to generate and identify other intergenotypic or interstrain heterodimers sharing the same N-terminal motif and focus on characterizing the nature of E1 and E2 cross-talk.

The Cross-talk between the N Terminus and tmd Highlights a New Role for Certain Amino Acids—It was surprising to find a need for compatibility and, therefore, maybe an interaction between the N terminus and tmd of E1 for H77. Indeed, even though the role of E1 is still poorly characterized and despite its interaction with E2 not yet being fully understood, these two E1 domains were not expected to interact, as the E1 N-terminal domain is localized to the intraluminal compartment and the other three amino acids identified lie in the transmembrane region. However, it can be speculated that the association of the AIL motif with the H77 E1 tmd may lead to an optimal conformation of E1 at a certain stage of the biosynthesis and thus be necessary for an optimal dialogue in the heterodimer E1E2 for the entry steps.

Previous studies have indicated that the tmd of HCV glycoproteins exhibits unusual features, although interestingly, the amino acids identified in the E1 tmd had not been previously

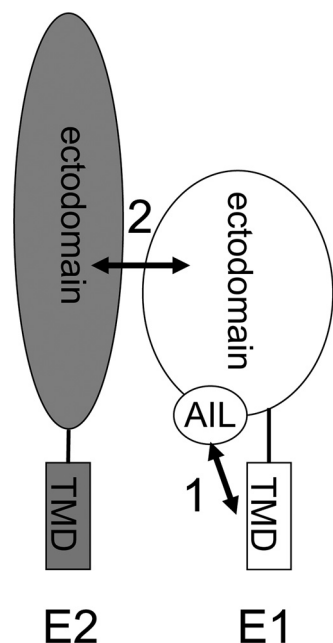


FIGURE 7. Schematic representation of potential interactions in E1E2 heterodimers necessary for entry. The transmembrane domains (TMD), the ectodomains of E1 and E2, and the N-terminal motif (AIL) of E1 H77 are represented. *Arrow 1* represents the interaction between the AIL motif and the tmd of H77 essential for entry in H77 heterodimer. *Arrow 2* represents the potential compensatory interaction that could act between Con1 E2 and H77 E1 ectodomain and allow entry without “interaction 1.”

highlighted (25, 26, 46). The tmd of E1 and E2 are composed of less than 30 amino acids forming two hydrophobic stretches separated by a short segment containing one or two fully conserved charged residues (47). A study of the topology of the tmd of HCV envelope glycoproteins has shown a reorientation of the C termini of these domains, leading to a single membrane-spanning topology (23). It can be speculated that at a certain point of synthesis and maybe also in later events, the amino acid identified here in the tmd may be exposed intraluminal and interact with the E1 N-terminal domain.

Another possibility stems from research showing that the amino acid sequence in the tmd or the intracytoplasmic tail can influence the conformation of the ectodomain (27, 48). Therefore, the amino acids identified in the H77 E1 tmd may have an indirect role in the N-terminal domain by allowing a particular conformation of the ectodomain and allowing the N-terminal domain to act efficiently.

Studies of HCV envelope glycoproteins with heterologous expression systems have shown that the tmd of these proteins plays a major role in the assembly of the E1E2 heterodimer (25) and its subcellular localization (46) and entry (26). Alanine insertions within the tmds of HCV envelope glycoproteins have identified the central regions of these domains as well as the N-terminal part of the tmd of E1 as sequences involved in heterodimerization (25). Another study identified individual residues within the central region as well as the N-terminal part of the tmd of E1 that participate in those interactions through a tryptophan replacement scan of these regions (26). Therefore, our studies attribute to the C-terminal half of the tmd a role in entry that has never been shown before and has no link with heterodimerization.

However, the role of the N-terminal domain of E1 is not known. Previous studies have never identified this region as important for E1E2 function. Indeed, only the tmd, juxtamembrane region (49), and the potential peptide fusion in E1 (16, 17) have been studied in depth. Our studies demonstrate that the AIL motif of E1 is important for the membrane fusion process (Fig. 5E), which probably explains the suboptimal titer of HCVpp (Fig. 5E) and the defect in proliferation of HCVcc H77MIM mutant. The conformation and the role of this region might give some clues about the role of E1 in E1E2 heterodimer during entry.

The Understanding of E1 Role during Entry Could Lead to New Therapeutic Strategies—Different strategies of vaccination have been considered, and some suggest the use of E1 as antigen (50–52). These assays only lead to a poor number of antibodies against E1, but some have the benefit of being able to cross-neutralize HCV entry of many strains. The epitope on E1 that has been characterized for these neutralizing antibodies and lies in the ectodomain of E1 (313–327) is outside our N-terminal region and the potential fusion peptide. As our motif is only important for H77, antibodies or peptides that could be synthesized against this region might not be efficient at blocking entry of different strains. Our results indicate, as the functionality of E1 is strain-dependent, that it would be difficult to find a clinical strategy that could prevent entry of HCV from different genotypes by targeting particular domains of E1.

To find the second potential interaction between E1 and E2 (Fig. 7), it would be interesting to study other strains that are not dependent on the AIL motif or have the AIL motif on both sequences to find another domain of E1 important for entry that could be more useful for different strains that could lead to the development of new therapeutic strategies.

Thus, these studies led to the identification of E1E2 domains that have co-evolved within a given HCV genotype and mediate the cross-talk necessary for achieving cell entry functions. The identified cross-talk is restricted to H77 and involves mainly E1. However, other interactions need to be identified to complement the proposed model, and domain interactions between E1 and E2 need to be more precisely determined.

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